THE INDEPENDENT PRODUCTION OF ATHEROSCLEROSIS AND THROMBOSIS IN THE RAT

G. A. GRESHAM AND A. N. HOWARD

From the Department of Pathology, University of Cambridge, The Dunn Nutritional Laboratory, University of Cambridge, and Medical Research Council

Received for publication February 27, 1960

IN recent years dietary fat has been implicated as a factor in both atherosclerosis and ischaemic heart disease. But the hypothesis is founded more on assumption than fact (Bronte-Stewart, 1958) and conclusive experimental proof is so far lacking.

Animal experiments play an important part in attempting to elucidate the nature of the diseases since it is not possible to determine satisfactorily the presence of atherosclerosis in living man. Furthermore, it is not justifiable to assume that the presence of ischaemic heart disease reflects the extent or degree of atherosclerosis (Morris, 1951 ; Robertson, 1959).

Thomas and Hartroft (1959) fed to rats a diet containing butter, cholesterol, cholic acid and thiouracil and produced myocardial infarction and vasothrombosis. They emphasized that such lesions might occur without any demonstrable vascular disease. The investigations reported here are concerned with the type of vascular lesions produced by diets containing variable amounts of either arachis oil or butter. The two fats differ in that arachis oil has a high content of linoleic acid and that butter contains saturated acids of low carbon number (Table I).

TABLE I.—*Proportions (per cent molar) of Fatty Acids in Butter and Arachis Oil**

	Number of C atoms	Number of C—C double bonds	Butter	Arachis oil
Butyric	4	0	10	—
Hexa-, Octa-, Decanoic and Lauric	6, 8 10, 12	0	15	—
Myristic	14	0	10	—
Palmitic	16	0	20	15
Stearic	18	0	10	5
Oleic	18	1	30	40–60
Linoleic	18	2	5	20–35

* Hilditch (1956).

EXPERIMENTAL

Animals.—Laboratory bred piebald rats were fed a stock diet after weaning until about 100 g. body weight and then placed into experimental groups. Each animal was housed in a separate cage, fed food and water *ad lib.* and weighed weekly.

TABLE II.—*Design of Experiment and Diets Used*

Group	Diet factor investigated	Number of animals group	Sex	Variable constituents in diet† (g.) Cholesterol	Thiouracil	Cholic acid	Butter	Arachis oil	Sucrose
1	Diet known to produce myocardial infarction	25	M.	50	3	20	400	—	169
2	Substitution of arachis oil for butter	20	„	50	3	20	—	400	169
3	Reduction of butter to 5 per cent	10	„	50	3	20	50	—	469
4	Substitution of females for males	15	F.	50	3	20	400	—	169
5	40 per cent butter alone*	10	M.	—	—	—	400	—	242
6	40 per cent arachis oil alone*	10	„	—	—	—	—	400	242
7	5 per cent butter alone*	20	„	—	—	—	50	—	592
8	Omission of thiouracil	10	„	50	—	20	400	—	172
9	Substitution of 10 per cent arachis oil for 40 per cent butter	10	„	50	3	20	—	100	469
10	10 per cent arachis oil only*	10	„	—	—	—	—	100	532

* i.e. cholesterol, cholic acid and thiouracil omitted.

† All diets included Casein 200, choline chloride 10, salts 40, cellulose powder 100, magnesium oxide 5, inositol 2 and B vitamins (thiamine 16 mg., riboflavin 16 mg.,pyroidoxine HCl, 16 mg., folic acid 10 mg., calcium pantothenate 40 mg., biotin 0·6 mg., B_{12} 0·05 mg., nicotinamide 0·2 g.).

TABLE III.—*Average Body Weight of Rats After* 5, 10 *and* 20 *Weeks on Experimental Regime, and Average Survival Times*

Group	Factor investigated	Number of animals/ group	Sex	Average body weight (g.) at (weeks) 0	5	10	20	Number surviving at 20 weeks	Average survival (days)
1	Diet known to produce myocardial infarction	25	M.	120	102	112§	—	0	95
2	Substitution of arachis oil for butter	20	„	102	102	97§	—	0	113
3	Reduction of butter to 5 per cent	10	„	102	122	—	—	0	44
4	Substitution of females for male	15	F	108	109	98§	—	0	72
5	40 per cent butter alone	10	M.	101	274	390‡	471*	10	*
6	40 per cent arachis oil alone	10	„	120	291	382‡	430*	10	*
7	5 per cent butter alone	20	„	102	250	316	366	10	*
8	Omission of thiouracil	10	„	102	221	275‡	313*	8	†
9	Substitution of 10 per cent arachis oil for 40 per cent butter	10	„	115	122	121§	—	0	100
10	10 per cent arachis oil cnly	10	„	103	253	334	401	10	*

* All survived.

† One died on 64th the other at the 56th day.

Statistics : Groups compared with group 10 and 20 weeks.

‡ $0{\cdot}05 > P > 0{\cdot}01$. § $P < 0{\cdot}001$.

Experimental design.—The diets fed are shown in Table II and were variations of that used by Thomas and Hartroft (1959) which was claimed to produce a high incidence of myocardial infarction. They were designed to investigate the following points: (i) the replacement of butter by arachis oil; (ii) the reduction of concentration, of butter to 5 per cent; (iii) replacement of males by females; (iv) the omission of cholesterol, cholic acid and thiouracil; (v) the omission of thiouracil alone. Animals receiving 5 per cent butter and 10 per cent arachis oil were used as controls. When a constituent of the diet was omitted, an equivalent weight of sucrose was added. The diets were prepared once weekly and in those containing a high concentration of fat, every attempt was made to avoid rancidity. Each rat was given one weekly supplement comprising 1000 i.u. vitamin A and 40 i.u. vitamin D, supplied as one drop of halibut liver oil, 2 mg. α-tocopheryl acetate in 2 drops arachis oil and 0·05 mg. 2-methyl-1 : 4 naphthoquinone in one drop arachis oil.

Electrocardiograms.—These were taken according to the method of Drury, Harris and Maudsley (1930) using an instrument of the mirror type manufactured by the Clifton Instrument Co., Cambridge. A standard lead (S2) and two unipolar leads (corresponding to the V1 and V2 positions in man) were taken of all animals in groups 1–4 fortnightly and of 5 animals in each of the groups 5–9 at least once a month.

Pathology.—Necropsy was made as soon as possible after death, the time interval varying from ½ hour to 3–4 days. If there was a time lag before death and necropsy, carcasses were stored in the refrigerator. Animals surviving 20 weeks were killed by ether anaesthesia. Specimens were taken of the heart, thoracic and abdominal aorta, kidneys, adrenals, liver, lungs, testes, skin, thyroid and spleen. Fixation was in all cases by neutral buffered formol saline. Frozen sections were stained by Oil red O and paraffin sections by a variety of conventional histological and histochemical methods in order to demonstrate special features.

Oil injection method.—Selected animals were killed by ether anaesthesia and the coronary vascular tree injected with radio opaque oil (neohydriol, iodized poppy-seed oil) by means of a cannula in the thoracic aorta; all other vessels coming off the aorta above the cannula were ligated. Using this method it was possible to study the macroscopic appearance of the coronary tree by X-ray photography and the microscopic appearance by frozen sections stained with oil red O; neohydriol is strongly sudanophil.

RESULTS

Body weights

The average weight of the different groups at 5, 10 and 20 weeks are given in Table III. Inclusion of thiouracil in the diet suppressed growth (groups 1–4 and 9) and the animals just maintained their body weight throughout the experiment. Rats consuming 40 per cent butter or 40 per cent arachis oil alone (groups 5 and 6) were obese and had higher body weights than the controls receiving 5 per cent butter and 10 per cent arachis oil alone (groups 7 and 10).

Mortality

As shown in Table II, animals in groups 1 and 2 survived for about the same time (14–15 weeks). Group 3 survived for only 6 weeks. Compared with animals in group 1, females (group 4) succumbed before males. Only those animals receiving thiouracil had a high mortality as in other groups most were living after 20 weeks.

Electrocardiograms

Compared with man the ST segment of the rat cardiogram is considerably elevated. Although a standard lead S2 and unipolar leads V1 and V2 were taken, it was found that unipolar lead V2 gave the most useful information. Among the experimental groups, abnormal patterns were seen in 5 animals out of 25 in group 1 (males) and 1 animal out of 15 in group 4 (females). Abnormalities consisted

of inverted T waves. As to be described later all animals with myocardial infarction had abnormal electrocardiograms. Such patterns were seen only in groups 1 and 4.

Pathology

In groups 1 and 4, the apex of the heart invariably showed a small patch of fatty material and occasionally a larger area of necrosis (Fig. 1). The lungs were congested and showed generalized oedema. In group 2, the patch at the apex of the heart was either minute or absent and the lungs were not congested.

Rats receiving cholesterol and cholic acid (groups 1–4, 8 and 9) had intensely fatty livers and the plasma contained a very high content of lipid so that vessels were often conspicuously white after death. As expected all animals receiving thiouracil had generalized symmetrical enlargement of the thyroid.

Microscopic examination

Occurrence of thrombosis.—In 8 animals in group 1 and 2 in group 4, large thrombi were seen in either the ventricles, atria, the thoracic aorta, or renal veins. Fig. 2 shows a thrombus in the left ventricle attached to an apical patch of lipid filled macrophages. Fig. 3 shows thrombi occurring simultaneously in the left atrium and left ventricle. Fig. 4 shows an endothelialized thrombus in the aorta and Fig. 5 in the renal vein. Thrombi were also attached to areas of infarction when these occurred (Fig. 6). No thrombi were seen in any group other than groups 1 and 4 (Table IV).

Occurrence of infarction.—Six animals in group 1 and one in Group 4 showed recent or partially healed myocardial infarction in either the left ventricular wall or ventricular septum. It appeared likely that luminal thrombi were the cause

TABLE IV.—*Distribution of Lesions*

Group	Description	Number of animals/ group	Number of animals showing lesions				
			Athero-sclerosis	Abundant endothelial lipid filled macrophages	Throm-bosis	Infarction	
						Heart	Kidney
1	Diet known to produce myocardial infarction	25	0	25	13	6	2
2	Substitution of arachis oil for butter	20	12	0	0	0	0
3	Reduction of butter to 5 per cent	10	0	0	0	0	0
4	Substitution of females for males	15	0	8	4	1	2
5	40 per cent butter alone	10	0	0	0	0	0
6	40 per cent arachis oil alone	10	0	0	0	0	0
7	5 per cent butter alone	10	0	0	0	0	0
8	Omission of thiouracil	10	0	2	0	0	0
9	Substitution of 10 per cent arachis oil for 40 per cent butter	10	3	0	0	0	0
10	10 per cent arachis oil only	10	0	0	0	0	0

of the infarction since organization in the thrombi was far advanced beyond that in the infarcts. Such thrombi produced infarction by either blocking ventricular-arterio-luminal vessels or, if present in the aorta by blocking coronary ostia. One rat which had an endothelialized thrombus in the aorta (Fig. 4) showed an abnormal electrocardiogram with an inverted T wave for some 4 weeks ; this became normal about a fortnight before death. It is possible that the aortic thrombus caused intermittent obstruction to coronary blood flow. All rats showing cardiac infarcts had abnormal electrocardiographic patterns.

A few animals were investigated using the oil injection method described above. No significant differences from normal were seen in group 1.

Two animals in group 1 and two in group 4 showed renal infarction. Both animals had thrombi in the renal veins ; it appeared likely that infarction was due to venous and not arterial thrombosis.

No other groups showed either myocardial or renal infarction (see Table IV).

Lipid changes in the vascular system

Groups 1 and 4 showed some accumulation of lipid filled macrophages particularly on the ventricular endocardial surface of the base of the mitral cusp (Fig. 7) and at the apex of the heart (Fig. 6). Small areas of intimal sudanophilia were seen in the abdominal aorta of 3 animals in group 1. This hardly amounted to atherosclerosis. Fig. 8 shows a typical section of aorta from this group and the absence of lipid infiltration.

In contrast, animals in group 2 showed atherosclerotic plaques in the thoracic aorta (Fig. 11) and coronary arteries (Fig. 9 and 10) which closely resembled those of human atherosclerosis (Fig. 12). We define atherosclerosis as a raised lesion capped by a fibro-elastic tissue beneath which is amorphous debris containing cholesterol crystals and other sudanophil lipid ; the underlying elastica being often fragmented. Lesions were seen mainly in the proximal aorta but none was seen in the abdominal aorta or renal arteries. Group 9, in which arachis oil was reduced from 40 per cent to 10 per cent was the only other group to show atherosclerotic lesions. The lesions were much smaller in size however and occurred in a smaller proportion of animals. No other groups showed similar changes (Table IV).

Lipid changes in the organs

Rats fed cholesterol and cholic acid (groups 1–4, 8 and 9) had large numbers of lipid filled macrophages in renal glomeruli, zona glomerulosa and reticularis of the adrenal, the red pulp of the spleen, in the pulmonary alveoli and in pleural tags of the lungs. Lipid in the lungs was found in all animals fed fat and probably arrived there by inhalation ; this is supported by the frequent presence of foreign body giant cells in relation to such lipid. The adrenal, of group 4 (females) showed more lipid than group 1 (males). The same groups of animals had intensely fatty livers and showed the presence of large numbers of cholesterol crystals when examined under ordinary light and polarized light. The deposition of cholesterol crystals was more extensive in group 2 than in the other groups. In addition, group 1 showed widespread hepatic cell vacuolation some of which was due to lipid material.

Other changes observed

Lungs from animals in groups 1 and 4 showed intense oedema, emphysema and congestion thus confirming the macroscopic findings. Other groups had moderate or no visible oedema.

Calcinosis of the arterioles of the thyroid and kidney were seen in all groups fed cholesterol and cholic acid. Two animals had large stones in the renal pelvis.

Thyroid glands.—Animals receiving thiouracil showed conspicuous hyperplasia. A curious finding was the occasional presence of colloid droplets in the acini.

DISCUSSION

In man, coronary thrombosis and atherosclerosis are usually associated clinically. Moreover it is recognized that the diseases are more prevalent in the obese (Mann and Stare, 1954). Our results indicate that thrombosis, atherosclerosis and obesity can be apparently produced independently using 3 different experimental regimes. A diet containing 5 per cent cholesterol, 2 per cent cholic acid, 0·3 per cent thiouracil and 40 per cent butter (Thomas and Hartroft, 1959) produced thrombosis, myocardial and renal infarction but no significant atherosclerosis. In contrast, replacement of the 40 per cent butter with 40 per cent arachis oil produced vascular lesions which closely resembled those of human atherosclerosis but no thrombosis or significant infarction. Animals fed 40 per cent butter alone or 40 per cent arachis oil alone became obese but developed none of these lesions.

But coronary thrombosis and atherosclerosis are more prevalent in countries with a high intake of animal fat (Bronte-Stewart, 1958). Sinclair (1956) has related the incidence to a relative deficiency of essential fatty acids which are present in only small amounts in most animal fats. Our results are difficult to reconcile with this hypothesis. Whilst it is true that the thrombosis was produced by the diet containing butter, atherosclerosis occurred only in those animals consuming arachis oil which contains a relatively high amount of essential fatty

EXPLANATION OF PLATES

FIG. 1.—Extensive infiltration of lipid at cardiac apex. Group 1.

FIG. 2.—Thrombus adherent to left ventricular apex. Group 1. H. & E. ×19.

FIG. 3.—Atrio-ventricular junction of heart showing mitral valve cusp diagonally placed with thrombus in left atrium (A) and in left ventricle (B). The ventricular septum is on the bottom right (C). Group 1. H. & E. ×19.

FIG. 4.—Endothelialized thrombus lying free in aorta. Group 1. H. & E. ×19.

FIG. 5.—Transverse section of renal pelvis showing thrombus in renal vein. Group 1. H. & E. ×29.

FIG. 6.—Thrombus adherent to left ventricular apex. Thrombus (T) in left ventricle; normal muscle (N); infarct (A); area of lipid infiltration (B). Group 1. H. & E. ×15.

FIG. 7.—Section through base of mitral cusp showing lipid filled macrophages on the ventricular endo-cardial surface. Group 1. H. & E. ×56.

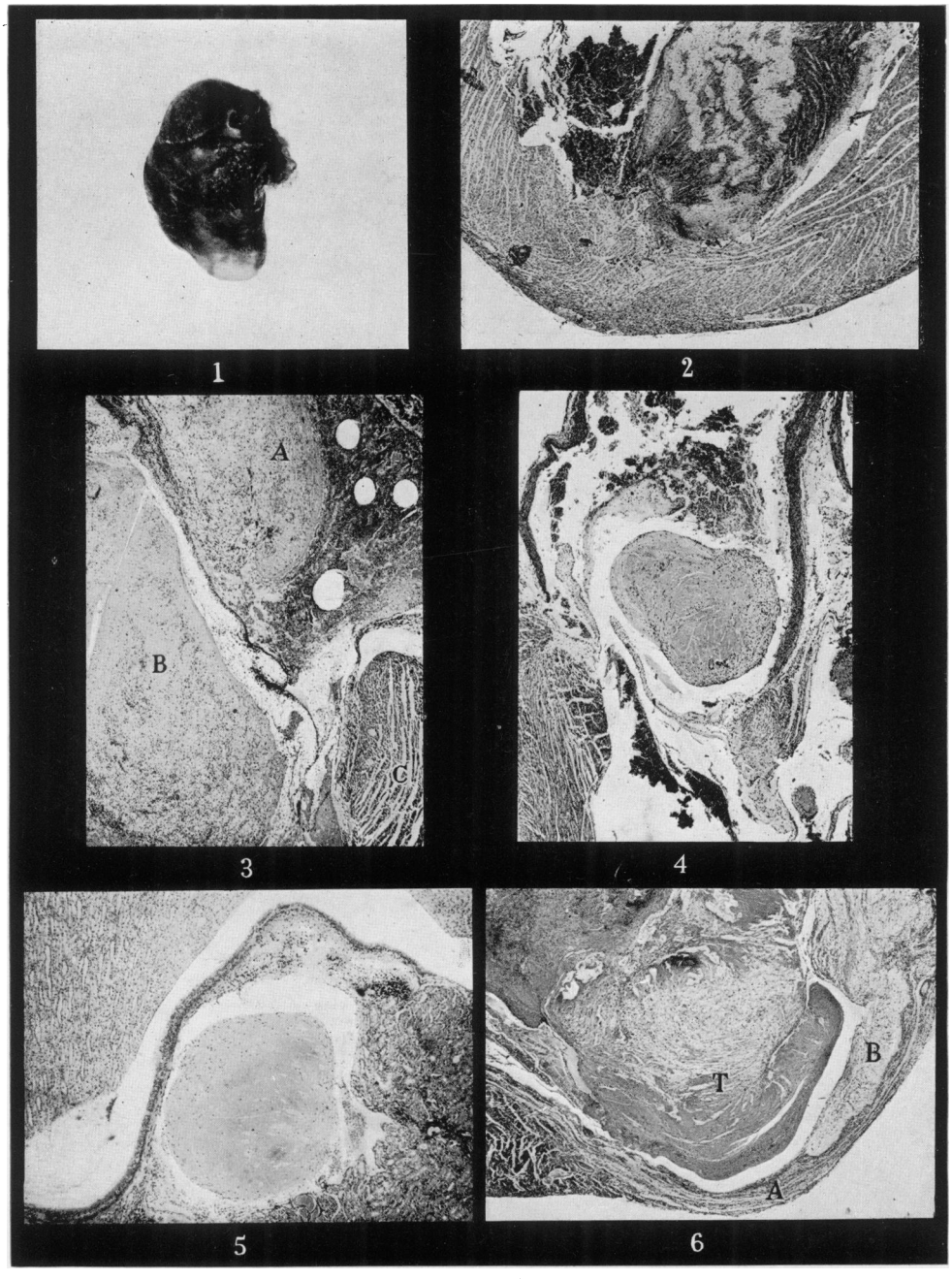
FIG. 8.—Aorta. Note one small area (W) of inner medial lipid infiltration. (M) Group 1. Oil red O. ×22.

FIG. 9.—Aorta (A) and coronary arteries (B and C) one of which (C) shows an atherosclerotic plaque. Group 2. H. & E. ×17.

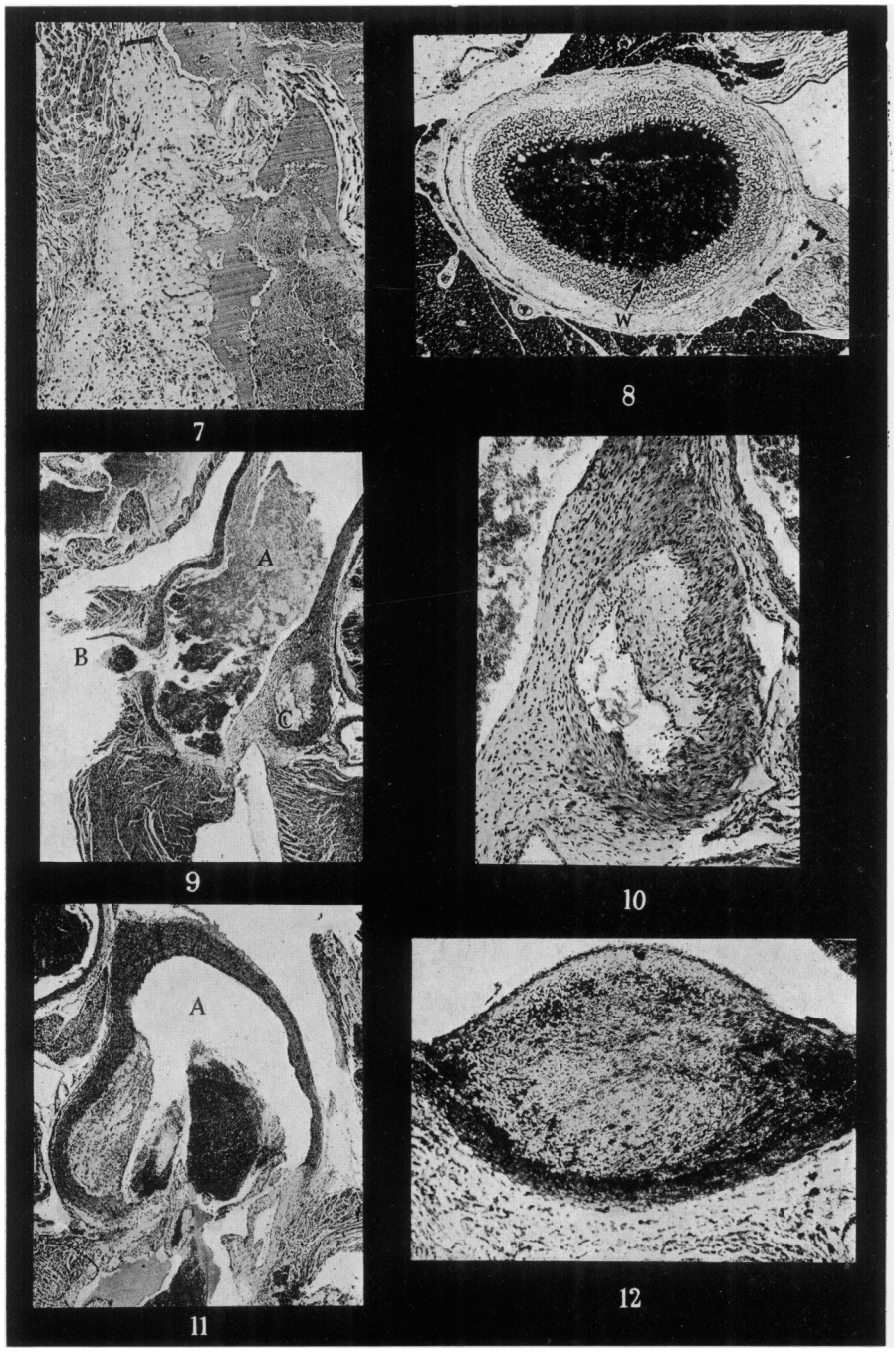
FIG. 10.—Coronary artery as shown in Fig. 9. Group 2. H. & E. ×52.

FIG. 11.—Aorta. (A) showing an atherosclerotic plaque. Group 2. H. & E. ×17.

FIG. 12.—Atherosclerotic plaque showing sudanophil lipid and a cap of fibro-elastic tissue. Oil red O. ×56.



Gresham and Howard.



7 8 9 10 11 12

Gresham and Howard.

acid, linoleic acid (25 per cent). Further work is obviously necessary to ascertain which constituents of either fat are responsible for the lesions observed.

Death in male rats fed the diet containing 40 per cent butter, cholesterol, cholic acid and thiouracil (group 1) was apparently the result of extensive thrombosis in the aorta, ventricles and atria leading to cardiac failure and subsequent congestion of the lungs. Myocardial infarction was probably related to the thrombosis and caused by obstruction to the arterio-luminal sinusoids. Failure to find arterial lesions constantly related to thrombi suggested that thrombosis was a result of changes in the properties of the blood either by an increase in coagulability or by decreased fibrinolysis. Moreover thrombosis was not confined to arteries but also involved the renal vein, and the obstruction caused thereby was no doubt responsible for the renal infarction observed. Electrocardiograms in rats with myocardial infarction were clearly abnormal and the technique recommends itself for use in future experiments since progress can be observed without killing the animal. More detailed studies of the electrocardiograms are to be presented elsewhere (Hill, Howard and Gresham, 1960).

All the above mentioned constituents in the diet appear to be essential for thrombosis. Thus the omission of thiouracil (group 8) or the reduction of the concentration of butter to 5 per cent (group 3) prevented thrombosis. Animals in the group with thiouracil omitted survived the experiment but the group in which the butter was reduced to 5 per cent lived only 6 weeks. Females (group 4) appeared to be less susceptible to thrombosis and to the infarction than males (group 2) but it should be noted that their survival time was somewhat less, and hence they were on the dietary regime for a shorter period.

The cause of death was not determined in the animals fed 40 per cent arachis oil, cholesterol, cholic acid and thiouracil. Cardiac failure seemed unlikely as the prime cause since the lungs were not usually congested and oedematous. A high intake of arachis oil apparently favoured the production of the atherosclerosis observed since fewer and less conspicuous lesions were seen when its concentration was reduced to 10 per cent (group 9). The atherosclerotic lesions closely resembled those seen in man and were not associated with thrombosis. Serum cholesterol values in the 2 groups showing atherosclerosis were not higher than the other groups (Howard and Gresham, unpublished). A characteristic finding, however, was a greater deposition of cholesterol in the liver than in other groups and the liver cells were packed with cholesterol crystals.

As might be expected in those groups consuming cholesterol and cholic acid, large quantities of lipid were taken up by phagocytic cells particularly in suprarenals, spleen, and lungs. Livers of these animals were intensely fatty and the lipid consisted chiefly of cholesterol esters. In many of these animals, arterial calcinosis occurred particularly in the kidney, thyroid and lungs. The mechanism of calcinosis appears obscure and would seem to warrant further study.

All the rats fed on diets containing 40 per cent butter alone, 40 per cent arachis oil alone, 5 per cent butter alone or 10 per cent arachis oil alone survived the experiment and some of these rats are being allowed to continue. It is hoped to give a more detailed report later. Animals examined after 4–6 months had developed no atherosclerotic or thrombotic lesions. It is difficult to extrapolate experimental data obtained in the rat to the occurrence of the diseases in man. Our data do not necessarily provide evidence that a high fat diet *per se* is the primary cause of atherosclerosis or ischaemic heart disease. On the other hand

it suggests that the type of fat consumed may have a major influence on the type of lesion produced.

SUMMARY

Rats were fed a diet containing 5 per cent cholesterol, 2 per cent cholic acid, 0·3 per cent thiouracil and variable amounts of butter or arachis oil. A pathological study was made of the heart, aorta and selected organs.

Only rats fed the above-mentioned diet containing 40 per cent butter had a high incidence of myocardial and renal infarcts and abnormal electrocardiograms. The infarction was attributed to thrombotic occlusion of arterioluminal sinusoids in the ventricles and of the renal veins in those with renal infarcts. Atherosclerosis was not seen.

Substitution of 40 per cent arachis oil for 40 per cent butter produced neither infarction nor thrombosis. In contrast, extensive atherosclerosis occurred in the thoracic aorta and coronary arteries and was similar to that seen in man. Atherosclerosis was less severe if the arachis oil content of the diet was reduced to 10 per cent.

Rats fed 40 per cent butter or 40 per cent arachis oil alone became obese but developed no vascular, renal or myocardial lesions.

All diets containing cholesterol and cholic acid produced arterial calcinosis and large numbers of lipid filled macrophages in most organs.

It was concluded that thrombosis, atherosclerosis and obesity are three independent phenomena.

We wish to thank M. A. Ward, Mr. S. G. Impey and Mr. A. J. King for their valuable technical assistance, and Mr. S. W. Patman for preparing the photomicrographs.

REFERENCES

Bronte-Stewart, B.—(1958) *Brit. Med. Bull.*, **14**, 243.

Drury, A. N., Harris, L. J. and Maudsley, C.—(1930) *Biochem. J.*, **24**, 1633.

Hill, R., Howard, A. N. and Gresham, G. A.—(1960) *Brit. J. exp. Path.*, In the Press.

Hilditch, T. P.—(1956) 'Chemical Constitution of Fats'. 3rd Ed. London (Chapman and Hall), pp. 129 and 230.

Mann, G. V. and Stare, F. J.—(1954) Symposium on atherosclerosis. National Research Council, Washington, D.C., publication No. 338, p. 169.

Morris, J. N.—(1951) *Lancet*, i, 1 and 69.

Robertson, W. B.—(1959) *Ibid.*, i, 444.

Sinclair, H. M.—(1956) *Ibid.*, i, 381.

Thomas, W. A. and Hartroft, W. S.—(1959) *Circulation*, **19**, 65.